Heparin Enhances the Inhibition of Factor Xa by Protein C Inhibitor in the Presence but Not in the Absence of Ca^{2+}†

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ABSTRACT: Protein C inhibitor (PCI) is a versatile serine protease inhibitor with both pro- and anticoagulant and other properties. Interactions of certain ligands with PCI, including heparin, affect its specificity for proteases. In this study, heparin was found to enhance PCI inhibition of factor Xa up to 42-fold in the presence of a physiological Ca^{2+} concentration, whereas no heparin-induced activation was observed in the absence of Ca^{2+}. These results thus show that factor Xa adds to the group of proteases whose inhibition by PCI is enhanced by heparin and that such inhibition contributes to the anticoagulant properties of PCI by a Ca^{2+}-dependent mechanism.

Protein C inhibitor (PCI)[1] is a multifunctional serine protease inhibitor, found in blood and other tissues, that belongs to the serpin superfamily of proteins (*1, 2*) and utilizes a typical serpin suicide mechanism for protease inhibition (*3*). The physiological role of PCI remains obscure, since it can inhibit many proteases. Among circulatory proteins, target proteases include activated protein C (APC), thrombin bound to thrombomodulin, free thrombin, factor Xa, factor XIa, plasma kallikrein, hepatocyte growth factor activator, urokinase, and tissue plasminogen activators (*4*−*7*). PCI is also required for reproduction and is found in high concentrations in seminal plasma, where it forms complexes with tissue kallikrein, prostate specific antigen, and acrosin (*8*−*10*). Furthermore, it can bind to several cofactor ligands and be internalized into cells (*11*−*13*). Due to the multiple activities of PCI, defining factors that contribute to bringing specificity to this serpin is of great medical interest.

Binding to ligands has been found to affect the ability of PCI to inactivate proteases. Heparin, for instance, activates the inhibitory potential of PCI toward several protease targets, including thrombin, APC, and acrosin (*10, 12, 13*), but does not activate PCI inhibition of plasma and tissue kallikrein and hepatocyte growth factor activator (*14*−*17*). GAGs can thus determine the activity as well as the specificity of PCI for proteases. The mechanism of GAG activation of PCI is not fully understood but is believed to involve a bridging effect, whereby formation of the protease−inhibitor complex is enhanced through the binding to the same heparin chain (*5*). Such a mechanism is supported by the crystal structure of the Michaelis complex of PCI, thrombin, and heparin, which suggested that the heparin-binding sites of thrombin and PCI are aligned and form a ternary complex with heparin (*18*). In a recent study, we demonstrated that PCI purified from human blood is highly heterogeneous and that the heterogeneity is caused by differences in N-glycosylation occupancy, N-glycan structures, and the presence of a form lacking the six N-terminal amino acids, denoted the Δ-6-N-cleaved form (*19*). We also showed that the N-terminus and the *N*-glycans of PCI affected the heparin- and thrombomodulin-enhanced rates of thrombin inhibition (*19*). Thus, in addition to ligand binding, posttranslational modifications of PCI affect its functions. In previous studies, it has been reported that PCI inhibition of factor Xa is not or only slightly affected by heparin (*12, 20*). Those studies were, however, conducted in the absence of Ca^{2+}. Here, we have determined kinetics for the rates of PCI inhibition of factor Xa in the absence or presence of Ca^{2+} and various heparin concentrations. A commercial human blood plasma-derived factor Xa fraction and two PCI forms isolated from human blood plasma were used, i.e., the Δ-6-N-cleaved form and the full-length form. Δ-6-N-cleaved PCI has a lower affinity for heparin than the full-length form (*19*). The results demonstrated that heparin enhanced PCI inhibition of factor Xa 14- and 42-fold for full-length and Δ-6-N-cleaved PCI, respectively, in the presence of Ca^{2+}. In contrast, no heparin enhancement was observed in the absence of Ca^{2+}.

EXPERIMENTAL PROCEDURES

Proteins and Saccharides. PCI was isolated from freshly frozen human blood plasma that originated from several healthy blood donors, as described previously (*21, 22*). Briefly, three consecutive affinity chromatography steps were employed, with the use of two immobilized monoclonal antibodies and one heparin-Sepharose chromatography step. The total PCI fraction was subsequently subjected to a more extended heparin Sepharose gradient, to separate the full-length form from the Δ-6-N-cleaved form (*19*). The homogeneity of the purified protein was investigated by 10−12%

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[1] Abbreviations: APC, activated protein C; GAG, glycosaminoglycan; k_{obs}, observed pseudo-first-order rate constant; k_2, second-order rate constant; PAGE, polyacrylamide gel electrophoresis; PCI, protein C inhibitor; RCL, reactive center loop; SDS, sodium dodecyl sulfate.

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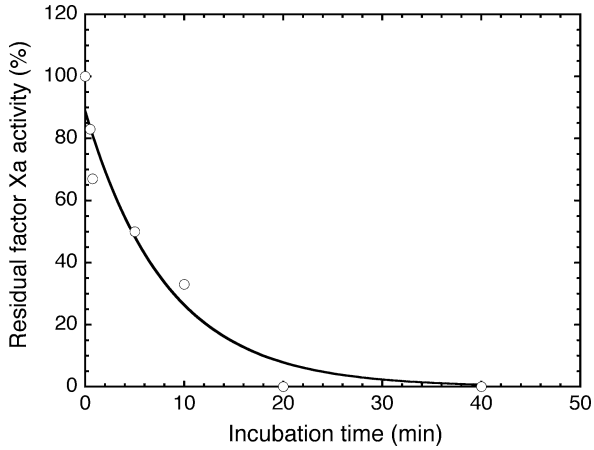

FIGURE 1: Rate of factor Xa inhibition by full-length PCI. The inhibition of factor Xa by PCI was measured as a function of time by a discontinuous assay, in the absence of heparin, as described in Experimental Procedures. The solid line represents the computer fit to a monophasic exponential decay function.

SDS−PAGE under reducing conditions with the Laemmli system. The gels were stained with Coomassie brilliant blue. The concentrations of the purified protein fractions were determined by amino acid analysis. The active concentrations of the PCI forms, determined as described previously (*19*), were used throughout this study.

Human factor Xa was purchased from Enzyme Research Laboratories (South Bend, IN). The active concentration, determined by titrations with 100% active human α-antithrombin (*23*), was used throughout this study.

Size-fractionated heparin with an average mass of ~11 kDa, derived from pig intestinal mucosa, was a gift from I. Björk (Swedish University of Agricultural Sciences, Uppsala, Sweden).

Kinetics of Factor Xa Inhibition. Second-order rate constants for the inhibition of factor Xa by the PCI variants were measured under pseudo-first-order conditions, using a discontinuous assay (*23*) in 20 mM Tris-HCl and 0.1 M NaCl at pH 7.4 and 25 °C. Factor Xa (final concentration of 10 nM) was incubated with a 10-fold excess of PCI. After different reaction times, varying between 10 s and 60 min, aliquots were diluted 10-fold in the assay buffer containing 165 μM substrate S2222 (Haemochrom Diagnostica AB, Mölndal, Sweden). The initial rates of the residual factor Xa activity were monitored in a dual-beam spectrophotometer at 405 nm and plotted against the incubation times. Observed pseudo-first-order rate constants, k_{obs}, were obtained by nonlinear least-squares fitting of such plots to a single-exponential decay function (*23*). Second-order rate constants were calculated by dividing k_{obs} by the active PCI concentration.

Effects of Heparin on the Rates of Factor Xa Inhibition. The rates of factor Xa inhibition were measured under the same conditions described above except that 0.058−38 μM heparin and 2.5 mM Ca^{2+} were added to the incubation mixtures. The dependence of k_{obs} on the heparin concentration was computer fit with eq 1 (*24*) with KaleidaGraph:

$$k_{obs} = k_{Xa}[PCI]_f + k_{H,Xa}[PCI\cdot Xa]\times\frac{K_{Xa,H}}{K_{Xa,H}+[H]_f} + k'_{H,Xa}[PCI\cdot H]\times\frac{[H]_f}{K_{Xa,H}+[H]_f} \quad (1)$$

where

$$[PCI\cdot H] = \left([PCI]_0 + [H]_0 + K_{PCI,H} - \sqrt{([PCI]_0 + [H]_0 + K_{PCI,H})^2 - 4[PCI]_0[H]_0}\right)/2$$

$$[PCI]_f = [PCI]_0 - [PCI\cdot H]$$

$$[H]_f = [H]_0 - [PCI\cdot H]$$

where $[PCI]_0$ and $[H]_0$ are the total PCI and heparin concentrations, $K_{Xa,H}$ and $K_{PCI,H}$ are dissociation equilibrium constants for the factor Xa−heparin and PCI−heparin interactions, and k_{Xa}, $k_{H,Xa}$, and $k'_{H,Xa}$ are second-order rate constants for the reactions of factor Xa with PCI, the PCI−heparin binary complex with factor Xa, and the factor Xa−heparin binary complex with the PCI−heparin binary complex, respectively. Measured k_{Xa} values, determined by the method described above (i.e., the k_2 values measured in the absence of cofactor), were used.

RESULTS

Kinetics for the Inhibition of Factor Xa by Full-Length and Δ-6-N-Cleaved PCI. Kinetics for PCI inhibition of factor Xa was studied by a discontinuous spectrophotometric assay, with the use of chromogenic substrate S2222, in the absence or presence of 2.5 mM Ca^{2+}. Pseudo-first-order conditions were employed with PCI concentrations 10-fold higher than those of factor Xa. Plots of residual factor Xa activity as a function of time were computer fit to a monophasic single-exponential decay function, as exemplified in Figure 1. Second-order rate constants for factor Xa inhibition in the absence of heparin and Ca^{2+} were (1.7 ± 0.2) and (0.39 ± 0.05) × 10^4 M^{-1} s^{-1} for the full-length and Δ-6-N-cleaved forms, respectively (Table 1). The corresponding values in the presence of Ca^{2+} were (1.0 ± 0.05) and (0.22 ± 0.06) × 10^4 M^{-1} s^{-1} for the full-length and Δ-6-N-cleaved forms, respectively (Table 1). These results demonstrate that Ca^{2+} somewhat lowered the rates of inhibition and that the full-length form inactivated factor Xa 4-fold more efficiently than the N-terminally cleaved form in the absence of heparin.

Effects of Heparin on the Rates of PCI Inhibition of Factor Xa. The effect of heparin on the rate of PCI inhibition of factor Xa was studied as described above, except that heparin was also added to the assay. Binding of heparin to PCI has previously been found to be unspecific with regard to the heparin sequence. Moreover, other GAGs and polyanions can bind to PCI and catalyze several of its inhibitory activities (*12, 13*). In this study, a size-fractionated 11 kDa heparin was used, containing ~36 monosaccharide residues. Such heparins efficiently catalyze other PCI−protease reactions (*12*). Moreover, similar heparins have previously been found to be sufficiently long to bridge antithrombin and factor Xa in a ternary complex (*25*). The rate of PCI inhibition of factor Xa increased in the presence of heparin when Ca^{2+} was added to the incubation mixture, whereas no increase in this rate was observed in the absence of Ca^{2+}. The dependence of the inhibition rates on the heparin concentration in the presence of Ca^{2+} followed a bell-shaped curve for both forms of PCI (Figure 2). This behavior is in agreement with a ternary complex mechanism, as suggested previously for the effect of heparin on PCI inhibition of other proteases, including thrombin, APC, and acrosin (*10, 12, 13, 26, 27*). Interestingly, both the height of the peak of the bell-shaped curve and the heparin concentration at which this peak was

Table 1: Rate Constants for Factor Xa Inhibition by Full-Length and Δ-6-N-Cleaved Forms of PCI in the Absence or Presence of Heparin and 2.5 mM Ca^{2+a}

PCI form		second-order rate constant (k_2) ($\times 10^4$ M^{-1} s^{-1})			
		k_2 in the absence of heparin[b]	optimal [heparin] (μM)	k_2 at optimal [heparin]	x-fold increase
full-length	without Ca^{2+}	1.7 ± 0.2			
	with Ca^{2+}	1.0 ± 0.05	1.1	14	14
Δ-6-N-cleaved	without Ca^2	0.39 ± 0.05			
	with Ca^{2+}	0.22 ± 0.06	2.3	9.3	42

[a] Pseudo-first-order rate constants, k_{obs}, were obtained by nonlinear regression fitting to a monophasic exponential decay function (Figure 1). The second-order rate constants at the maximal heparin concentration were calculated from the highest points in the computer fits of Figure 2. [b] Mean values ± the standard error of two or three measurements.

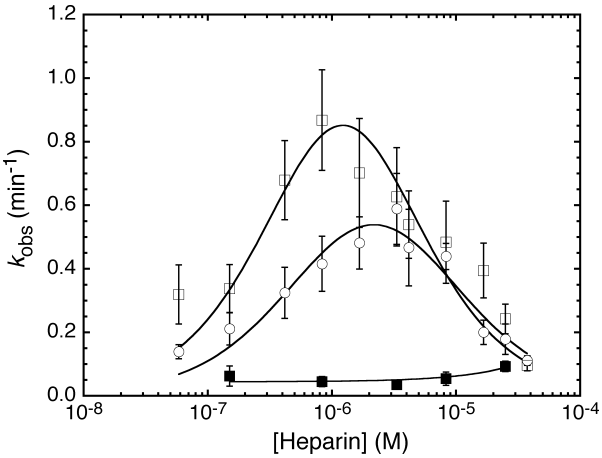

FIGURE 2: Effects of heparin on PCI inhibition of factor Xa in the absence or presence of Ca^{2+}. The inhibition of factor Xa was measured by a discontinuous assay, at different heparin concentrations: (■) total PCI without Ca^{2+}, (□) full-length PCI with Ca^{2+}, and (○) Δ-6-N-cleaved PCI with Ca^{2+}. k_{obs} values (±standard error) were obtained by nonlinear regression fitting to a monophasic exponential decay function (shown in Figure 1). Solid lines represent computer fits to eq 1.

observed differed for the full-length form compared with the Δ-6-N-cleaved form. The optimal heparin concentrations were thus 1.1 and 2.3 μM and the maximal second-order rate constants (k_2) in the presence of optimal heparin concentrations 14 and 9×10^4 M^{-1} s^{-1} for the full-length and Δ-6-N-cleaved forms, respectively (Table 1).

DISCUSSION

This study was undertaken to determine the effects of heparin on the inhibition of factor Xa by two forms of PCI isolated from human blood plasma. Differences between these plasma-derived PCI forms have been described previously (*19*). The Δ-6-N-cleaved form lacks the N-terminal residues HRHHPR (*22*). This sequence of PCI magnifies the heparin- and thrombomodulin-enhanced rates of thrombin inhibition (*19*). In contrast to thrombin inhibition, however, this sequence was found to affect PCI inhibition of factor Xa not only in the presence but also in the absence of cofactors, as revealed by the 4-fold lower uncatalyzed rate of factor Xa inhibition by Δ-6-N-cleaved versus that by full-length PCI. The 2-fold higher heparin concentration required for the Δ-6-N-cleaved form versus that required for the full-length form to reach maximal cofactor-enhanced inhibitory rates is in agreement with a lower affinity of the N-terminally cleaved form than the full-length form for heparin. The difference in maximal heparin-catalyzed rates for the two forms of PCI was somewhat lower (1.5-fold) than the difference in the uncatalyzed rates, because the enhancing effect of heparin in the presence of Ca^{2+} was 42-fold for the Δ-6-N-cleaved form and 14-fold for the full-length form. These findings suggest that the N-terminal region of PCI not only affects the heparin-enhanced rate of factor Xa inhibition by stabilizing the ternary complex due to a higher affinity for heparin but also directly affects the mechanism of PCI inhibition of factor Xa.

The heparin binding properties of factor Xa are altered by Ca^{2+}, which binds to the negatively charged γ-carboxyglutamic acid domain of the protease, enabling factor Xa to expose its exosite for heparin binding (*25*). Ca^{2+} has thus previously been found to affect the heparin-enhanced rate of antithrombin inhibition of factor Xa by promoting the assembly of an intermediate heparin−antithrombin−factor Xa bridging complex (*25, 28*). In contrast to PCI, heparin enhances antithrombin inhibition of factor Xa by both an allosteric and bridging mechanism. The bridging effect is present only to a small extent in the absence of Ca^{2+} but increases in the presence of Ca^{2+} (*28, 29*). The Ca^{2+}-induced heparin enhancing effect on antithrombin inhibition of factor Xa (*25*) is highly similar to the 14-fold Ca^{2+}-induced heparin enhancement observed here for the rate of factor Xa inhibition by full-length PCI. Thus, Ca^{2+} presumably affects the heparin-enhanced PCI inhibition of factor Xa by a bridging mechanism similar to that for antithrombin (*25, 28*). A similar Ca^{2+} effect has been observed for heparin activation of PCI inhibition of APC (*30, 31*) and has been suggested to contribute to the highly variable values published for the heparin-enhancing effect of PCI inhibition of APC. The reported values for PCI inhibition of APC in the absence and presence of heparin are, however, highly variable even in the absence of Ca^{2+} (*12, 20*), suggesting that there are additional reasons for the inconsistency in reported values for the kinetics of PCI inhibition of APC. Apart from the circulation, heparin or similar GAGs are believed to affect the functions of PCI in, for instance, the reproductive tract. The inhibitory rate of PCI inhibition of acrosin is enhanced by heparin and follows a bell-shaped dependence on the heparin concentration, reaching a maximal 230-fold activation at the optimal heparin concentration (*10*).

PCI was originally identified as a procoagulant through its ability to inhibit APC (*32*) but appears to have both pro- and anticoagulant and other properties (reviewed in ref *9*). The biological functions of PCI have been difficult to determine due to the lack of appropriate animal models. Whereas PCI is expressed in several human tissues, mice and rats express PCI only in the reproductive organs.

Therefore, transgenic mice expressing PCI in several tissues have been generated (*33*). The importance of PCI in reproduction is evident from the PCI knockout mice, which have impaired spermatogenesis and are infertile (*34*). A role for PCI in human reproduction is supported by the correlation between low levels of PCI or dysfunctional PCI in seminal plasma and infertility (*35, 36*). There is, however, clinical support also for the importance of PCI in hemostasis. For instance, PCI levels are decreased in certain patients with intravascular hypercoagulation (*37, 38*). The procoagulant role of PCI is emphasized by the reported increases in the levels of the APC−PCI complex in patients with cardiovascular disorders (*39*−*42*). Intriguingly, an anticoagulant role of PCI is also supported by animal studies that reported improving effects of PCI on hypercoagulability states, such as disseminated intravascular coagulation (*43, 44*) and pulmonary hypertension (*45*). The dual functions of PCI in hemostasis may be medically important, because if this serpin can act as a pro- or anticoagulant, depending on the coagulability state, it may be a good drug candidate for conditions where there is a combined risk of bleeding and thrombus formation, for instance, disseminated intravascular coagulation.

The maximal heparin-enhanced inhibitory activity of PCI toward factor Xa described here is on the same order of magnitude as that toward thrombin described previously (*19*). PCI exists at a much lower concentration than antithrombin in the circulation, and the rates of PCI inhibition of thrombin and factor Xa in vitro are dramatically slower than those of antithrombin. However, the inhibitions of both thrombin and factor Xa are presumably factors contributing to the anticoagulant properties of PCI during, for instance, hypercoagulability states, in consideration of the improving effects of PCI under such conditions (*43*−*45*). In contrast to antithrombin, no specific sequence requirements for heparin or heparan sulfate binding to PCI have been identified (*12*), suggesting that the latter serpin is able to bind to many different heparan sulfate or other GAG sequences found on the vessel walls. Moreover, in a previous study, we demonstrated the presence of sialyl $Le^{a/x}$ epitopes on the N-linked glycans of PCI (*19*). These epitopes are known to bind to selectins, which are expressed in, for example, endothelial cells during inflammation. It is thus possible that PCI may be locally enriched on the endothelial surface to enable efficient protease inhibition at specific sites, which may compensate for the relatively low concentration of this inhibitor in the blood.

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